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<p>(54) Title: MONOCLONAL ANTIBODIES TO ANTIGENS EXPRESSED BY HUMAN DENDRITIC CELLS (57) Abstract The present invention relates to monoclonal antibodies reactive with human dendritic cells. In particular, it relates to one monoclonal antibody directed to B70 (or B7-2 or CD86) which is expressed by human dendritic cells, methods of generating the antibody, and methods of using the antibody. Antibodies directed to antigens that are expressed preferentially or at higher levels by dendritic cells than by other blood cells have a wide range of applications, including but not limited to, dendritic cell isolation, detection of such cells in normal and pathological tissues, and inhibition of antigen presentation and T cell activation by dendritic cells.</p>		

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MONOCLONAL ANTIBODIES TO ANTIGENS
EXPRESSED BY HUMAN DENDRITIC CELLS

1. INTRODUCTION

5 The present invention relates to monoclonal antibodies reactive with human dendritic cells. In particular, it relates to one monoclonal antibody directed to B70 (or B7-2 or CD86) which is expressed by human dendritic cells, methods of generating the antibody, and methods of using the
10 antibody. Antibodies directed to antigens that are expressed preferentially or at higher levels by dendritic cells than by other blood cells have a wide range of applications, including but not limited to, dendritic cell isolation, detection of such cells in normal and pathological tissues,
15 and inhibition of antigen presentation and T cell activation by dendritic cells.

2. BACKGROUND OF THE INVENTION

 The introduction of a foreign antigen into an individual
20 elicits an immune response consisting of two major components, the cellular and humoral immune responses, mediated by two functionally distinct populations of lymphocytes known as T and B cells, respectively. The T cells may be further divided into two subsets by function and
25 phenotype. A subset of T cells responds to antigen stimulation by producing lymphokines which "help" or activate various other cell types in the immune system. Another T cell subset is capable of developing into antigen-specific cytotoxic effector cells, being able to directly kill
30 antigen-positive target cells. On the other hand, the B cell response is primarily carried out by secretory proteins, antibodies, which directly bind and neutralize antigens.

 A salient feature of both T and B cell responses is their exquisite specificity for the immunizing antigen;
35 however, the mechanisms for antigen recognition differ between these two cell types. B cells recognize antigens by antibodies, either acting as cell surface receptors or as

secreted proteins, which bind directly to antigens on a solid surface or in solution, whereas T cells only recognize antigens that have been processed or degraded into small fragments and presented on a solid phase such as the surface of antigen-presenting cells (APC). Additionally, antigenic fragments must be presented to T cells in association with major histocompatibility complex (MHC)-encoded class I or class II molecules. CD4⁺ T cells recognize antigens presented by MHC class II products, while CD8⁺ T cells recognize antigens in the context of MHC class I.

The presentation of antigens to T cells is carried out by specialized cell populations referred to as APC. These cells are involved in early events of an immune response, thus they are critical to the initiation of both T and B cell responses. Typically, APC include macrophages/monocytes, B cells, and bone marrow-derived dendritic cells (DC). DC are sometimes also referred to as "professional" APC. APC are capable of internalizing exogenous antigens, cleaving them into smaller fragments in enzyme-rich vesicles, and coupling the fragments to MHC-encoded class I or class II products for expression on the cell surface (Goldberg and Rock, 1992, Nature 357:375-379). Since APC express both MHC-encoded class I and class II glycoproteins, they can present antigenic fragments to both CD4⁺ and CD8⁺ T cells for the initiation of an immune response.

By definition, APC not only can present antigens to T cells with antigen-specific receptors, but can provide all the signals necessary for T cell activation. Such signals are incompletely defined, but probably involve a variety of cell surface molecules as well as cytokines or growth factors. Further, the factors necessary for the activation of naive or unprimed T cells may be different from those required for the re-activation of previously primed memory T cells. The ability of APC to both present antigens and deliver signals for T cell activation is commonly referred to as an accessory cell function. Although monocytes and B cells have been shown to be competent APC, their antigen

presenting capacities in vitro appear to be limited to the re-activation of previously sensitized T cells. Hence, they are not capable of directly activating functionally naive or unprimed T cell populations.

5 The term "dendritic cells" refers to a diverse population of morphologically similar cell types found in a variety of lymphoid and non-lymphoid tissues (Steinman, 1991, Ann. Rev. Immunol. 9:271-296). These cells include lymphoid DC of the spleen, Langerhans cells of the epidermis, and
10 veiled cells in the blood circulation. The vast majority of published reports have utilized DC isolated from the mouse spleen, which show that DC are unique APC in that they are capable of activating naive T cells in primary antigen-specific response. (Inaba et al., 1987, J. Exp. Med.
15 166:182-194; Hengel et al., 1987 J. Immunol., 139:4196-4202; Kast et al., 1988, J. Immunol., 140:3186-3193; Romani et al., 1989, J. Exp. Med. 169:1169-1178; Macatonia et al., 1989, J. Exp. Med. 169:1255-1264; Inaba et al., 1990, J. Exp. Med. 172:631-6640). Therefore, it is possible that human DC are
20 also capable of such potent antigen presentation function.

 Recently, a few studies have described the isolation of human DC from the peripheral blood. (Young and Steinman, 1990, J. Exp. Med. 171:1315-1332; Freudenthal and Steinman, 1990, Proc. Natl. Acad. Sci. USA 87:7698-7702; Macatonia et
25 al., 1989, Immunol. 67:285-289; Markowicz and Engleman, 1990, J. Clin. Invest. 85:955-961). The human peripheral blood-derived DC are shown to be superior APC than monocytes in activating secondary T cell responses (Van Voorhis et al., 1983, J. Exp. Med. 158:174). However, their enrichment
30 procedures often require a series of tedious density gradient centrifugation steps, sheep red cell rosetting and antibody selection.

 There remains a need for monoclonal antibodies (MAb) directed to antigens selectively expressed by human DC that
35 may be used to facilitate their isolation. Such antibodies may also be useful in identifying DC in tissue sections. Furthermore, such MAb may recognize molecules that are

involved in antigen presentation and T cell activation, thus they may also be useful in inhibiting DC-T cell interactions, particularly the induction of primary T cell responses. The difficulty in the generation of antibodies to DC is, in part, 5 due to the difficulty in obtaining large numbers of these cells for use as immunogens, since they are present in low quantities in human tissues. There is also little knowledge in regard to specific molecules in these cells that contribute to their potent APC function.

10

3. SUMMARY OF THE INVENTION

The present invention relates to MAb directed to antigens expressed by human DC, methods of generating the antibodies and methods of using the same.

15 The invention is based, in part, on Applicants' discovery that human DC may be enriched from the peripheral blood and prepared as immunogens to immunize animals for the generation of MAb. The hybridomas obtained are first screened for their secreted antibodies to bind to DC, but not 20 to other fresh peripheral blood mononuclear cells. After cell cloning, the MAb are further characterized by their reactivity with various blood cell populations. Five MAb have been produced by this method, which bind to human DC with varying degrees of specificity. One such MAb designated 25 IT-209 is of particular interest because of its binding pattern in brightly staining DC, but not resting T cells, B cells, monocytes. Although this antibody also reacts well with certain Epstein Barr Virus-transformed lymphoblastoid cell lines, it binds poorly to the majority of normal blood 30 cells tested.

Furthermore, IT-209 is shown to be capable of inhibiting both allogeneic T cell reactivity and antigen-specific T cell proliferation induced by autologous DC, indicating that it is directed to a molecule involved in a critical step of antigen 35 presentation and/or T cell activation. In addition, IT-209 inhibits DC presentation of exogenous antigens to naive T cells. The antigen recognized by IT-209 is shown to be a co-

stimulatory molecule known as B70 (CD86). A wide variety of uses for such a MAb are encompassed by the invention described herein, including but not limited to, the isolation of human DC for in vitro priming or re-activation of T cells, the detection of DC in normal and pathological tissues, and the inhibition of undesirable immune responses such as in allergy, autoimmunity and transplant rejection.

4. BRIEF DESCRIPTION OF THE DRAWINGS

- 10 FIG. 1A, MAb in ascites inhibit CD4⁺ T cell
1B, and proliferation in autologous MLR. 5×10^4
1C CD4⁺ T cells are incubated with 5×10^3 DC. Each
experiment was performed in quadruplicate samples. ■
= positive control, ▨ = 1/100, ▩ = 1/1,000, ▧ =
15 1/10,000, □ = 1/100,000.
- FIG. 2A, MAb in ascites inhibit T cell proliferation
and 2B in allogeneic MLR. ■ = 1/100, ▨ = 1/1,000, ▩ =
20 1/10,000.
- FIG. 3A IT-209 inhibits autologous antigen-specific CD4⁺ T
cell proliferative response to M tuberculosis at
5 μg/ml.
- 25 FIG. 3B IT-209 inhibits autologous antigen-specific CD4⁺ T
cell proliferative response to PPD at 20 μg/ml
- FIG. 3C IT-209 inhibits autologous antigen-specific CD4⁺ T
30 cell proliferative response to enterotoxin A was at
1 ng/ml.
- FIG. 4. Purified IT-209 inhibits autologous (5×10^3 DC + 5×10^4 CD4⁺ T Cells) and allogeneic (500 DC + 5×10^4 CD4⁺ T Cells) MLR in a dose dependent manner. An isotype-matched IgM anti-Leu7 antibody is used as
- 35

negative control. ○ = IT-209 + allo, △ = IT-209 + auto, ● = Leu7 + allo, ▲ = Leu7 + auto.

FIG. 5A- IT-209 inhibits primary CD4⁺ T cell
5 5C. proliferative responses to soluble antigens presented by autologous DC. 10⁵ CD4⁺ T cells were cultured with irradiated antigen-pulsed autologous APC, including either 10⁴ monocytes activated by plastic adherence or 2 x 10³ DC (5A,B), or graded
10 numbers of APC (5C), in the presence of the indicated MABs. Isotype-matched MABs of irrelevant specificity were added to control cultures. Results represent the mean ± SEM of quadruplicate cultures. In 5C, SEM were always <10% and were
15 omitted for clarity. The data are representative of four experiments.

FIG. 6A- IT-209 specifically reacts with B70-
6D. transfected L cells. CD80-(6A) and B70 (6B)-
20 transfected L cells were stained with FITC-conjugated L307 (anti-CD80), IT2.1 (anti-B70), IT-209 and FITC anti-mouse IgG1 control MAB. For binding inhibition studies, B70-transfected L cells were pre-incubated with 10 µg of purified IT2.1 or
25 IT2.2 MAB for 20 min before the addition of FITC-labeled MABs, followed by staining with FITC-conjugated IT-209 (6C) or IT2.2 (6D). Immunofluorescence and flow cytometry were done using a FACScan. Data are displayed as histograms.
30 The x-axis represents fluorescence and the y-axis the relative cell number. Histograms from cells stained with control (cont.) antibody (dotted lines) are superimposed over the histograms of cells stained with anti-CD80, anti-B70 and IT-209,
35 as indicated.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to MAb to antigens expressed by human DC, methods of generating such antibodies and uses of such MAb.

5 The MAbs described in the following section are generated against purified DC membrane extracts. An antibody designated IT-209 is selected by its preferential binding to DC, and subsequently shown to be specific for a T cell costimulatory molecule expressed by DC. The target antigen
10 is known as B70 (or B7-2) which is recently renamed as CD86 (Azuma et al., 1993, Nature 366:76; Freeman et al., 1993, Science 262:909; Hathcock et al., 1993, Science 262:905; Chen et al., 1994, J. Immunol. 152:4929). Since IT-209 inhibits naive T cell activation by DC, the CD28/B70 pathway is shown
15 to be involved in DC dependent CD4⁺ T cell activation. However, the results also show that blockade of the interactions between CD28 and its ligands reduces but does not totally eliminate either primary or secondary T cell responses to soluble antigens, thus other signalling pathways
20 may also be involved.

Although the specific procedures and methods described herein are exemplified using mouse MAb generated against DC isolated from human blood, they are merely illustrative for the practice of the invention. Analogous procedures and
25 techniques are equally applicable to a variety of animal hosts for producing MAb and human DC isolated from any tissue where they are found.

5.1. PREPARATION OF HUMAN DENDRITIC CELLS AS IMMUNOGENS

30 In order to generate MAb to antigens preferentially expressed by human DC, there are two major hindrances that must first be overcome. The first relates to the low quantities of DC in human tissues and thus they must be
35 enriched to sufficient quantities and in relatively pure form for use as immunogens. Although DC are found in both lymphoid and non-lymphoid tissues, a natural and easily

accessible source of DC in man is the peripheral blood; however, human blood only contains an estimate of fewer than 1 DC per 100 white blood cells. Secondly, there must be an efficient method for differential screening of the specific antibodies desired, i.e., to select for antibodies that bind strongly to DC but less so to other blood cells. For the purpose of the instant application, DC are defined as large mononuclear cells, capable of APC function, which express high levels of MHC class I and II antigens (HLA-DR, DQ and DP), but lack the traditional lineage markers of T, B, monocyte and natural killer cell lineages such as CD3, CD14, CD19, CD20, and CD16.

Although the potency of the accessory cell function of DC in antigen presentation allows for the use of these cells in relatively small numbers when enriched, it is preferred that they be enriched to >50% for use as immunogens. Human DC may be isolated from any tissues where they reside, using a variety of separation methods. Example 6, *infra*, presents variants of such methods as illustrations for isolating DC from human peripheral blood. In accordance with this aspect of the invention, human peripheral blood mononuclear leukocytes (PBML) may be isolated from blood samples, particularly buffy coats or leukocytes prepared by apheresis, by "FICOLL HYPAQUE" gradient centrifugation (FICOLL, Pharmacia Fine Chemicals AB, Uppsala Sweden) followed by "PERCOLL" density centrifugation (Markowicz and Engleman, 1990, J. Clin. Invest. 85:955) and "METRIZAMIDE" gradient centrifugation. It is also important that DC are allowed to differentiate in order to exhibit their potent antigen presenting function, such as by overnight culture in teflon dishes. DC are in the low buoyant density (LD) fraction of the "METRIZAMIDE". The LD fraction can then be subjected to second "METRIZAMIDE" (Sigma Chemical Co.) gradient to obtain a further enriched population of DC. DC may be enriched using additional protocols, depending on the level of purity required.

Alternatively, DC may be isolated by procedures involving repetitive density gradient centrifugation, positive selection, negative selection, or a combination thereof. For example, the human PBML may be subjected to 5 centrifugation in "NYCOPREP 1.068" (Nycomed Pharma AS, Oslo, Norway). Positive selection does not necessarily require the use of antibodies that recognize DC-specific determinants. For example, B cells and monocytes may be depleted first from the DC-containing fraction after density 10 gradient centrifugation, plastic adhesion, Fc receptor panning, nylon wool, and antibody-coated beads, then an antibody to MHC-Class II antigen can be used to positively select for DC. Negative selection includes modifications of the protocol disclosed herein. For example, a DC-containing 15 cell preparation may be reacted with one or more antibodies directed at cell surface antigens not expressed by DC for the removal of non-DC. Antibodies to any T cell, B cell, monocyte, and granulocyte markers may be used. Examples of such antibodies include anti-CD3, anti-CD4, and anti-CD8 20 specific for T cells; anti-CD19 and anti-CD20 specific for B cells; anti-CD14 specific for monocytes; and anti-CD16 specific for natural killer cells. These antibodies may be applied in any combination repeatedly or in a sequential manner for the enrichment of DC. Upon binding to the 25 antibodies, the cells may be removed by adsorption to a solid surface coated with an anti-mouse antibody, as the majority of monoclonal antibodies directed at cell surface markers are of mouse origin, or if the antibodies are conjugated with biotin, the antibody-bound cells can be removed by an avidin 30 or streptavidin-coated surface; or if the antibodies are conjugated to magnetic beads, the cells expressing antigens recognized by the antibodies can be removed in a magnetic field (Harlow and Lane, 1988, "Antibody", Cold Spring Harbor Laboratory).

35

5.2. ANTIBODY PRODUCTION

Various methods may be used to produce antibodies which recognize novel antigenic markers expressed by human DC. Any procedure known in the art may be used for the production of antibodies to these cells after they have been isolated. For the production of antibodies, various host animals can be immunized by injection with viable, purified or partially purified DC, fixed cells or membrane preparations, including, but not limited to, those of rabbits, hamsters, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

MAb which are substantially homogeneous antibodies to single antigenic epitopes on DC may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 495-497), the more recent human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Monoclonal antibodies can be screened differentially by selective binding to DC, but not or only weakly to freshly isolated or activated granulocytes, monocytes, T cells, B cells and stem cells.

Antibody fragments which contain the binding site of the molecule may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the

antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab'), fragments.

A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine MAb and a human immunoglobulin constant region. Techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. USA 81:6851; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. This approach is particularly useful if the antibodies are administered into humans. Chimeric antibodies present less xenogeneic epitopes in inducing an anti-mouse Ig response when injected in man.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-425; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce DC-reactive single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragment of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Additionally, the whole antibody molecule or its Fab, F(ab'), or Fv fragment may be conjugated to any of a variety of compounds including, but not limited to, signal generating compounds such as a fluorochrome, radio-isotope, a chromophore, an enzyme, a chemoluminescent or bioluminescent molecule, etc. Alternatively, the whole antibody or its Fab, F(ab'), or Fv fragment may be conjugated to growth factor which may enhance or inhibit the biological activity of DC; or to toxins so that DC which express the corresponding antigens would be selectively killed (Vitetta and Uhr, 1985, Annu Rev. Immunol. 3:197). Methods which can be used for

conjugating labels, proteins, toxins etc. to antibodies and antibody fragments are well known in the art (See, for example, U.S. Patent Nos. 4,220,450; 2,235,869; 3,935,074 and 3,996,345).

5

5.3. USES OF MONOCLONAL ANTIBODIES TO DENDRITIC CELLS

A variety of uses of MAb to DC are encompassed by the present invention. An antibody exhibiting specificity for human DC in that it does not bind to freshly isolated T cells, B cells, NK cells, granulocytes, monocytes, red blood cells and platelet, may be used to isolate DC in a one step affinity cell separation procedure. Antibodies to markers that are selectively or preferentially expressed by DC, i.e., certain but not all blood cells also express it, may still be used effectively in combination with other methods such as density gradient centrifugation to substantially reduce the time-consuming and cumbersome procedures currently employed for the isolation of DC.

For the practice of this aspect of the invention, a MAb may be conjugated to fluorochromes such as fluorescein isothiocyanate and used to select for DC from a cell mixture by flow cytometry using a fluorescence activated cell sorter or may be conjugated to biotin for use in biotin-avidin or biotin-streptavidin separations. In the latter method, avidin or streptavidin is bound to a solid support such as affinity column matrix or plastic surfaces. In addition, antibodies may be coated with magnetic beads, reacted with a cell mixture, and the antibody-bound DC removed by a magnetic field.

Furthermore, such MAb may be conjugated to an enzyme for use in immunohistochemistry. For example, certain disorders, especially autoimmune diseases, may be induced or sustained by an aberrant function of DC in excessive antigen presentation and T cell activation. Thus, the number of DC in the affected tissues may increase and the detection of a change in DC quantities in tissues may be of diagnostic value

for such disease conditions. Alternatively, certain immunodeficiency states may be caused by a decrease in DC numbers or abnormal DC function, thus MAb may be applied on tissue sections to enumerate DC numbers or to detect the expression of certain APC-associated molecules.

MAb may be generated against DC molecules that are involved in antigen presentation function. For example, antibody IT-209 disclosed herein in Example 6, infra, is capable of blocking the activation of T cells by DC by disrupting CD28/B70 interactions. Thus, this antibody may be used to treat DC in vitro or in vivo to inhibit the initiation or re-stimulation of an immune response. This aspect of the invention is particularly useful in preventing undesirable immune responses in autoimmunity, transplantation rejection and allergy. Although other antibodies have been produced against the B70 molecule, IT-209 is unique in its specificity and function. The experiments described in Example 6.2 show that IT-209 recognizes a distinct epitope from those by other antibodies (Azuma et al., 1993, Nature.336:76). In addition, IT-209 does not bind resting monocytes, while other antibodies do. Moreover, IT-209 blocks B70 interaction with its ligand, but certain other reported anti-B70 antibodies do not. (Engel et al., 1994, Blood 84:1402, Nozawa et al., 1993, J. Pathol. 169:309). Thus, IT-209 may be particularly useful for both DC isolation and inhibition of T cell function.

For in vivo administration of MAb, it is preferable that such antibodies be converted to chimeric antibodies in an effort to reduce human anti-mouse Ig responses. The antibodies may be administered using techniques well known to those in the art. Preferably, agents are formulated and administered systemically. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences", 18th ed., 1990, Mack Publishing Co., Easton, PA. Suitable routes may include oral, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as direct

- intraventricular, intravenous, or intraperitoneal, injections, just to name a few. Most preferably, administration is intravenous. For injection, the MAb of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.
- Effective concentrations and frequencies of dosages of MAb to be administered may be determined through procedures well known to those in the art, which address such parameters as biological half-life, bioavailability, and toxicity. A preferred dosage concentration may range from about 0.1 mg/kg body weight to about 20 mg/kg body weight, with about 10 mg/kg body weight being most preferred. Because antibodies typically exhibit long half-lives in circulation, a single administration of MAb may be sufficient to maintain the required circulating concentration.
- Additionally, MAb directed to DC molecules that are involved in the specific antigen presentation function of DC may be used to isolate and identify the genes encoding such molecules. Antibodies may be used for screening expression libraries made from DC for the molecular cloning of the coding sequences (Seed and Aruffo, 1987, Proc. Natl. Acad. Sci. USA 84:3365-3369).

6. EXAMPLE: GENERATION OF MONOCLONAL ANTIBODIES TO HUMAN DENDRITIC CELLS

6.1. MATERIALS AND METHODS

6.1.1. CELL SEPARATION

Human DC were obtained from buffy coats of healthy donors. PBML were isolated by "FICOLL-HYPAQUE" gradient centrifugation (Boyum, 1968, Scand. J. Clin. Lab. Invest: 21:21-29). To separate monocytes from the remaining mononuclear cells, the preparation was further fractionated

over a four layer discontinuous "PERCOLL" gradient (30%, 40%, 50.5% and 75% by diluting stock isotonic "PERCOLL" at 1.129 g/ml with Dulbecco's phosphate buffered saline containing 5% human serum), performed by centrifugation at 2400 rpm for 20 min at 4°C, the monocyte-depleted mononuclear cell fraction (T cells, B cells, NK cells, and DC) was collected from the 75% layer (Markowicz and Engleman, 1990, J. Clin. Invest. 85: 955-961). The cells ($3-7 \times 10^5$ /50 ml of RPMI containing 10% pooled human serum) were then cultured overnight in teflon vessels to facilitate DC differentiation and thereafter the cultured cells were subjected to gradient centrifugation in "METRIZAMIDE" (Sigma Chemical Co.) by overlaying them onto 10 ml of 15.5% (wt/vol) "METRIZAMIDE" followed by centrifugation at 1800 rpm for 10 min at room temperature. The DC enriched in the low density fraction of the "METRIZAMIDE" were further enriched over a second "METRIZAMIDE" gradient (14%) in a similar manner. The purity of the DC obtained using this procedure was 60-90%. It should be noted that for purposes of preparing DC, other methods known in the art could be used in place of "METRIZAMIDE" gradients (Markowicz and Engleman, 1990, J. Clin. Invest. 85:955; Van Voorhis et al., 1983, J. Exp. Med. 158:174).

6.1.2. IMMUNIZATION AND CELL FUSION

The partially enriched human DC were resuspended at 5×10^6 cells/ml in hypotonic Tris buffer made up of 10 mM Tris, 1 mM EDTA and 50 μ M PMSF. The cells were allowed to swell for 0.2 hour on ice and then lysed by passing through a 25 gauge needle several times and centrifuged at 15,000g. The precipitate represented a crude membrane preparation which was subsequently resuspended in 500 μ l of a solution of 20 mM Tris/150 mM saline, 1% Nonidet P-40 and 50 μ M PMSF and allowed to sit at 2°C on ice. The detergent extract was then cleared by ultracentrifugation at 10,000 g to remove non-soluble debris and nuclei. The solubilized membrane extracts were then treated with a mixture of antibodies which reacted with immunogenic antigens known to be expressed by DC. These

included antibodies to HLA-DR (CA-141 from ATCC), HLA-DQ (Leu10 from Becton Dickinson), HLA-class I (W632 from ATCC) and LFA-1 (TS1 from ATCC) antigens. All antibodies were used at 10 µg/ml. The resulting immune complexes were cleared from the extracts by incubation with protein G-Sepharose beads (Zymed, South San Francisco, CA). The immunodepleted extracts containing soluble DC membrane proteins were then adsorbed onto 0.7 micron latex beads (Interfacial Dynamics Corp.) by overnight incubation. The beads were washed in PBS and resuspended in 500 µl of PBS. 250 µl of the suspension were injected into normal female BALB/c mice intraperitoneally. The animals were boosted three weeks later in the same manner.

The mice were tested for their serum reactivity in binding to DC and boosted with 1×10^6 intact DC about two months after the last immunization. Three days later, the mice were sacrificed and their spleen cells fused with SP2/0 myeloma cells using polyethylene glycol (Kohler and Milstein, 1975, Nature 256:495). The resultant hybridomas were grown in HAT medium and screened for the secretion of antibodies in culture supernatants that were reactive with human DC but not with freshly isolated human peripheral blood mononuclear cells (PBMC). The hybridomas that satisfied this staining characteristic were cloned by limiting dilution four times and further analyzed. The cloned hybridoma cells were injected into syngeneic pristine-primed BALB/c mice for the production of ascites.

6.1.3. FLOW CYTOMETRY ANALYSIS

For antibody staining experiments, 10^5 - 10^6 cells were collected and washed in cold PBS containing 1% BSA and 0.1% sodium azide. The cell pellets were suspended in 100 µl of the same buffer containing hybridoma supernatants at 4°C for 30 minutes. Cells were then washed in cold PBS twice and incubated with a second step fluorescein conjugated antibody specific for mouse IgG (Zymed, South San Francisco, CA) or mouse IgM (American Qualex, La Mirada, CA) for an additional

30 minutes. After washing, the cells were resuspended in 0.5 ml of paraformaldehyde and analyzed by an EPICS Profile II Flow Cytometer (Coulter Electronics, Hialeah, FL.). For direct staining, MAb conjugated to FITC or PE were purchased from Becton Dickinson (San Jose, CA), except for FITC-conjugated IgM of irrelevant specificity, which was purchased from Coulter Corp. (Hialeah, FL).

6.1.4. MIXED LEUKOCYTE REACTIONS (MLR) AND T CELL PROLIFERATION

10 Partially purified DC in RPMI medium supplemented with 10% human serum were plated in microtiter plates and irradiated at 2,500 rads from a cesium-137 source. Thereafter, T cells were added to the wells in culture medium and incubated at 37°C in 10% CO₂ in air for 6 days. For MLR
15 assays, 5×10^4 T cells were incubated with 500 DC in allogeneic stimulation, or with 5×10^3 DC in autologous stimulation, whereas 10^5 purified CD4⁺ T cells were added to 1-2 $\times 10^3$ DC pulsed with various concentrations of specific
20 antigens for inducing antigen-specific T cell proliferation. The antigens used were M. tuberculosis extract, purified protein derivative (PPD) and staphylococcus aureus enterotoxin A (Sigma Chemical Co.). The DC were pulsed with antigens at 37°C for at least 3 hours prior to the addition
25 of T cells. The cultures were then pulsed with 1 μ ci [³H] thymidine per well for 16 hours and harvested with a MASH apparatus (Otto Hiller Co., Madison, NJ) for determination of [³H] thymidine incorporation. For the antibody blocking studies, serial dilutions of the MAb ascites were added to
30 the wells at the initiation of culture. DC were also suspended in 1 μ g/ml of IgG MAb or in 5 μ g/ml of IgM MAb or with 10 μ g/ml of CTLA-4-Ig at 4°C for 30 minutes before the addition of CD4⁺ T cells. Anti-Leu 7 specific for CD7 (Becton-Dickinson Corp., San Jose, CA) was used as negative
35 control.

6.1.5. CELL LINES

T cell leukemia lines, myeloid cell lines, B cell lymphoma lines, and EBV transformed lines were obtained either from the American Type Culture Collection (ATCC, 5 Rockville, MD) or from the 10th International Histocompatibility Workshop. Cell lines were cultured in complete medium and split one day prior to immunofluorescence analysis. CD4⁺ or CD8⁺ T cell lines or clones were generated as described previously (Rivas et al., 1990, J. Immunol 10 145:470), CD4⁺ or CD8⁺ T cells were alloactivated with EBV cell lines (irradiated 100 Gy) and restimulated weekly. The alloactivated cells were propagated as T cell lines and expanded in conditioned medium supplemented with IL-2 containing supernatant.

15 Murine L cell fibroblasts were transfected with human CD80 cDNA (Azuma et al., 1992, J. Exp. Med. 175:353) and B70 (or CD86) cDNA (Azuma et al., 1993, Nature 366:76) in the BCMGS_{neo} expression vector as described (Azuma et al., 1992, J. Immunol. 149:1115). Stable transfectants were obtained by 20 growth in 0.4 mg/ml G418 (Sigma, St. Louis, MO) and selected for expression of CD80 or B70 using flow cytometry.

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6.1.6. CELL ACTIVATION

The low density fraction of cells obtained at the interface between 40% and 50% PERCOLL, containing more than 90% CD14⁺ cells, was extensively washed and cultured in complete medium at 2×10^6 cells/ml. In some experiments, the cells were allowed to adhere to petri dishes at 37°C for 30 min in complete medium containing 2.5% human serum and the nonadherent cells were then removed by vigorous washing with warm RPMI 1640. This procedure left a monolayer of adherent monocytes that was subsequently cultured in 10 ml of complete medium at 37°C with or without 500 U/ml of rIFN- γ . B cells were activated by coculture for 2 to 3 days with irradiated (25 Gy) allogeneic CD4⁺ T cells, previously plated in 12 well plates coated with OKT3 (anti-CD3) MAb in medium containing 10 ng/ml of rIL-4. T cells were activated by culturing PBMC for 10 days in petri dishes coated with purified anti-CD3 MAb (OKT3) with addition of 100 U/ml of rIL-2 at the fifth day of culture. NK cells were activated by culture for three days in medium containing 500 U/ml of rIL-2.

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6.2. EXAMPLES

In order to generate monoclonal antibodies to antigens preferentially expressed by human DC, human PBML were subjected to a series of enrichment steps to obtain a partially purified population of DC for use in immunization. In brief, the buffy coats of human blood were first isolated by "FICOLL HYPaque" to obtain PBMC. These cells were then further separated on a four layer "PERCOLL" discontinuous gradient followed by overnight culture in teflon dishes and "METRIZAMIDE" centrifugation. This method produced a final cell population that was highly enriched for DC. More than 85% of the final cell population stained with MAb to HLA-DR or CD80, but not with MAb to CD3, CD14, CD16 or CD19.

The DC were lysed and membrane extracts were prepared as immunogens. Prior to immunization, the extracts were reacted with antibodies specific for several strongly immunogenic antigens known to be expressed by DC in an effort to favor

host antibody production against novel antigens preferentially expressed on DC membrane by first removing the commonly known antigens. After fusion, five hybridomas were cloned and selected for further studies due to binding activities in their supernatants to DC as determined by flow cytometry. The five MAb are designated IT-1, IT-53, IT-131, IT-194 and IT-209. All MAb are IgM except for IT-1 which is an IgG. All five antibodies stained human peripheral blood DC brightly (mean channel fluorescence 20-80) and at least half of 30 Epstein Barr Virus-transformed lymphoblastoid cell lines (LCL) tested. However, the reactivity pattern of the antibodies differed in their staining of other blood cells (Table I). For example, antibody IT-1 stained all activated T and B cells. IT-131 stained activated T cells. IT-53 and IT-194 both stained resting B cells. IT-209 exhibited the most restricted staining pattern of the five antibodies. It should be noted that antibody staining was measured in relative terms; therefore, negative reactivity of an antibody with a particular cell population does not necessarily imply that there is no expression of the antigen at all, albeit its expression is quantitatively lower than in other cells.

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TABLE I

ANTIBODY DESIGNATION	ISOTYPE	REACTIVITY				
		DC	EBV-LCL	ACTIVATED T	RESTING B*	MONOCYTES*
IT-1	IgG	+	+	+	-	-
IT-53	IgM	+	+	-	+	-
IT-131	IgM	+	+	+	-	-
IT-194	IgM	+	+	-	+	-
IT-209	IgM	+	+	-	-	-

* Freshly isolated PBMC analyzed by two color flow cytometry with an antibody to a lineage specific marker and a test antibody described herein.

Antibody IT-209 bound strongly to DC (mean channel fluorescence 50-70) but not to CD3⁺ T cells, CD14⁺ monocytes, PHA-activated T cells or CD19⁺ B cells as determined by flow cytometric analysis. However, it stained several LCL with a mean channel fluorescence of 8-20. When one-color analysis of monocytes was performed, IT-209 staining remained within the negative gate, but showed a shift of its peak to higher fluorescence intensity, as compared to the control. Adherent monocytes became IT-209 positive within 2-3 hours and reached full expression within 24 hours of culture. IT-209 expression was only slightly enhanced by adding interferon- γ to the cultures. Freshly isolated monocytes also failed to stain with an antibody to CD80 and, in contrast to staining with IT-209, high concentrations of interferon- γ were required to induce CD80 expression on these cells as reported (Freedman et al., 1991, Cell. Immunol. 137:429).

Table II summarizes the detailed staining pattern obtained with IT-209 on a panel of PBMC and cultured cell lines. A fraction (<20%) of T cells activated with OKT3 in combination with exogenous IL-2 stained with IT-209 after 10 days of culture. However, the percentage was low by comparison to that of CD80 positive T cells (60-80%). Several CD4⁺ and CD8⁺ alloreactive T cell clones were negative for IT-209. Fresh peripheral blood B cells were negative for IT-209 staining, whereas B cells stimulated by irradiated allogeneic CD4⁺ T cells which had been activated by solid phase OKT3 MAb and cultured in medium containing recombinant IL-4 were positive. NK cells isolated by positive selection and cultured for 3 days in medium containing 500 U/ml of IL-2 were negative. Finally, non-lymphoid blood components, such as platelets, red blood cells and granulocytes also failed to stain with IT-209. The majority of EBV-transformed LCL and Burkitt lymphoma cell lines stained brightly with IT-209, while only three of these lines (Ramos, ST486 and Namalwa) were negative. Of a series of myeloid cell lines examined, only THP1 was clearly

positive. All T leukemia cell lines tested were negative except for two HTLV-I infected cell lines (HUT-102 and MT-2).

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Table II IT-209 Expression Pattern

Cells from PBMC					
	Mono- cytes	T	B	NK	DC
% of IT-209+ among resting or freshly isolated cells*	<5	<5	<5	<5	<5
% of IT-209+ among activated or cultured cells*	>80	10-25	>65	<5	>90
Cell Lines					
	IT-209+¶			IT209-	
B lines	REM, Arent, SKF, PGF, KHY, MSAB, LBF, CCRF, SB, NC37, CESS, RAJI, JIYOYE, DAUDI, JY, 9037, 9059, 9062, 9064, 721.221			RAMOS, NAMALWA, ST486	
T lines	MT-2, HUT-102			MOLT3, MOLT4, PEER, HPBALL, HUT78, VB, H9, HSB2, JURKAT, CEM	
Myeloid lines	THP1, K562			KG1a5, HL60, HEL, U937	

* Cells were purified and cultured or activated as described in Section 6.1.6.

¶ IT209+ refers to staining of more than 20% of the cells.

Antibody IT-209 is of particular interest not only because of its preferential binding to DC, it also inhibited T cell activation by DC. When dilutions of IT-209 ascites were added to cultures of freshly isolated CD4⁺ T cells and DC, the T cell response was inhibited to a variety of stimuli, including autologous MLR, (FIG. 1A, 1B and 1C), allogeneic MLR (FIG. 2A and 2B), and antigen-specific T cell responses to M. tuberculosis extract (FIG. 3A), PPD (FIG. 3B), and S. aureus enterotoxin A (FIG. 3C). These results indicate that IT-209 recognizes an antigen that is highly and preferentially expressed by human DC, and this antigen may be involved in critical events during antigen presentation by DC to T cells.

IT-209 was subsequently purified from ascitic fluid and shown to inhibit both autologous and allogeneic MLR in a dose dependent manner (FIG. 4). In the antibody inhibition experiments, an anti-HLA-DR MAb (L243) was the most potent inhibitor of the MLR, whereas irrelevant MAb of IgM and IgG isotypes had no effect. An anti-CD28 MAb also inhibited the DC induced MLR to a similar degree as IT-209, whereas an anti-CD80 MAb had only a slight effect. Moreover, the addition of IT-209 and an anti-CD80 MAb to the same culture did not result in incremental inhibition beyond that mediated by IT-209 or anti-CD28 alone. Similar results in terms of percent inhibition of proliferation were obtained by using CTLA-4-Ig instead of anti-CD28 MAb.

KLH and recombinant HIV gp160 proteins to which the vast majority of healthy individuals have not been sensitized, were chosen for the purpose of testing the primary proliferative response of CD4⁺ T cells to soluble antigens. CD4⁺ T cells did not respond to these antigens in the presence of monocytes, including adherent, B70⁺ monocytes (FIG. 5A, 5B and 5C). However, CD4⁺ T cells from the same donors proliferated significantly when KLH (FIG. 5B and 5C) or HIV gp160 (FIG. 5A) were presented by autologous DC. As shown, the responses to these antigens were substantially reduced (83 ± 5% inhibition) in the presence of IT-209, anti-

CD28 or anti-HLA-DR, and were only marginally affected by anti-CD80 MAb ($30 \pm 5\%$ inhibition). The degree of inhibition was constant and not related to the number of DC in culture (FIG. 5C). None of the irrelevant, isotype matched MAbs had 5 any significant effects on these responses.

One cell surface molecule with expression characteristics similar to that of the IT-209 antigen was known as the B7 antigen. However, recent studies identified another related T cell co-stimulatory molecule, referred to 10 as B7-2 (B70), thus B7 is now more appropriately known as B7-1 (or CD80). CD80 is thought to have co-stimulatory activity and its expression is believed to be essential for antigen presentation. The CD80 antigen is known to be expressed by DC, activated T cells, activated B cells and activated 15 monocytes. In contrast IT-209 did not stain activated T cells and only weakly stained monocytes activated by interferon- γ . Furthermore, preincubation of DC or LCL with IT-209 did not inhibit subsequent binding of fluorochrome-conjugated anti-CD80 antibody. Therefore, IT-209 does not 20 recognize the CD80 antigen.

To directly address the similarities between the antigen recognized by IT-209 and CD80 (B7-1) and B70 (B7-2), murine L cells expressing either CD80 or B70 as a consequence of transfection with specific cDNAs were evaluated for staining 25 with IT-209. As shown in FIG. 6A-6D, IT-209 reacted strongly with L cells transfected with B70 cDNA (FIG. 6A) but not with cells transfected with CD80 cDNA (FIG. 6B), whereas an anti-CD80 MAb (L307) stained L cells transfected with CD80 cDNA (FIG. 6B) but not with B70 cDNA (FIG. 6A). An irrelevant 30 isotype-matched MAb failed to stain any of the transfected cells. These results indicate that IT-209 recognizes the B70 antigen but not CD80. IT-209 staining of B70 transfected L cells was not affected by preincubating the same cells with IT2.1 MAb, one of two MAb used in the original expression 35 cloning of B70 cDNA (FIG. 6C). By contrast, IT-209 staining was reduced, although incompletely, by pretreating the transfected L cells with another anti-B70 MAb IT2.2 (FIG.

6C). Furthermore, as shown in FIG. 6D, IT2.1 MAb did not prevent IT2.2 MAb from binding to B70. Taken together, these data indicate that these three MABs recognize different epitopes on the B70 antigen. Interestingly, while IT2.1 stained resting monocytes, IT-209 did not.

7. DEPOSIT OF CELL LINE

The following hybridoma cell line was deposited with the American Type Culture Collection, Rockville, Maryland and assigned the following accession number:

<u>Hybridoma</u>	<u>ATCC Accession Number</u>
IT-209	ATCC HB 11498

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A monoclonal antibody which binds selectively to differentiated human dendritic cells, but does not bind 5 freshly isolated monocytes, T cells, B cells, and natural killer cells.
2. The monoclonal antibody of Claim 1 which also inhibits human T cell activation by allogeneic dendritic 10 cells or antigen-pulsed autologous dendritic cells.
3. The monoclonal antibody of Claim 2 which is directed to B70 antigen.
- 15 4. The monoclonal antibody of Claim 3 which is produced by hybridoma IT-209 as deposited with the ATCC having an accession number HB11498.
- 20 5. A monoclonal antibody, the antigen-binding region of which competitively inhibits the immunospecific binding of monoclonal antibody produced by hybridoma IT-209 as deposited with the ATCC having an accession number HB11498.
- 25 6. A biologically active fragment of the monoclonal antibody of Claim 5.
7. The monoclonal antibody of Claim 5 which is conjugated to a label capable of producing a detectable 30 signal.
8. The monoclonal antibody of Claim 7 in which the label is a fluorochrome or an enzyme.
- 35 9. A method of enriching human dendritic cells comprising reacting a mixed population of cells with a monoclonal antibody produced by hybridoma IT-209 as deposited

with the ATCC having an accession number HB11498, and removing the antibody-bound cells from unbound cells.

10. A method of inhibiting human T cell activation
5 by dendritic cells in an individual comprising administering
an effective amount of a monoclonal antibody produced by
hybridoma IT-209 as deposited with the ATCC having an
accession number HB11498.

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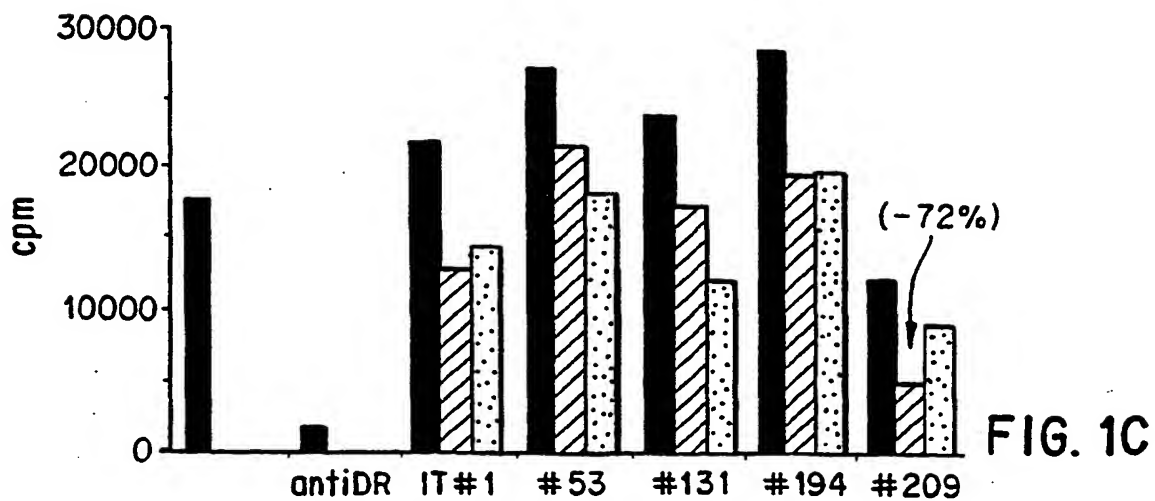
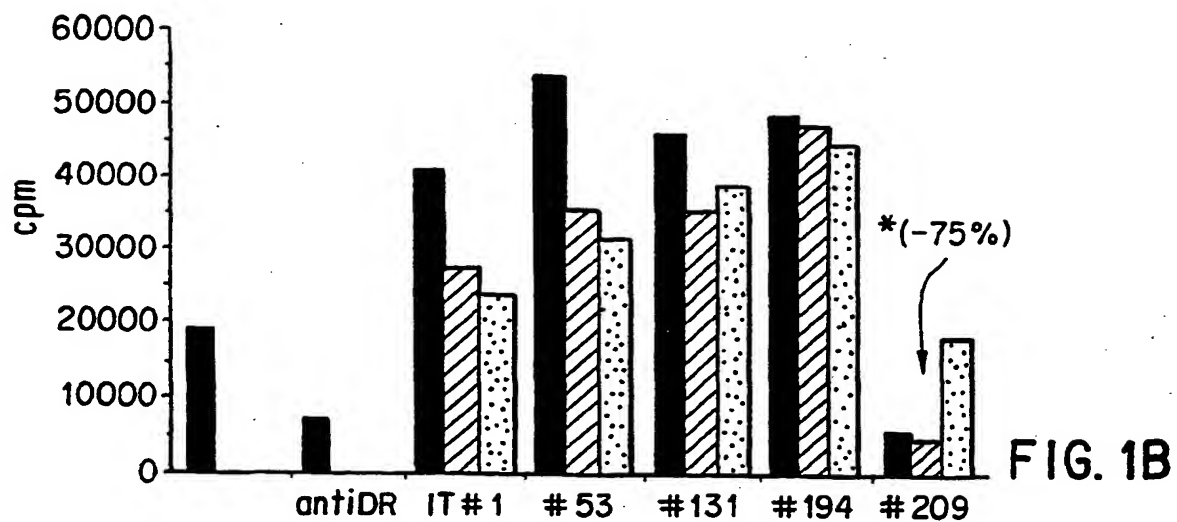
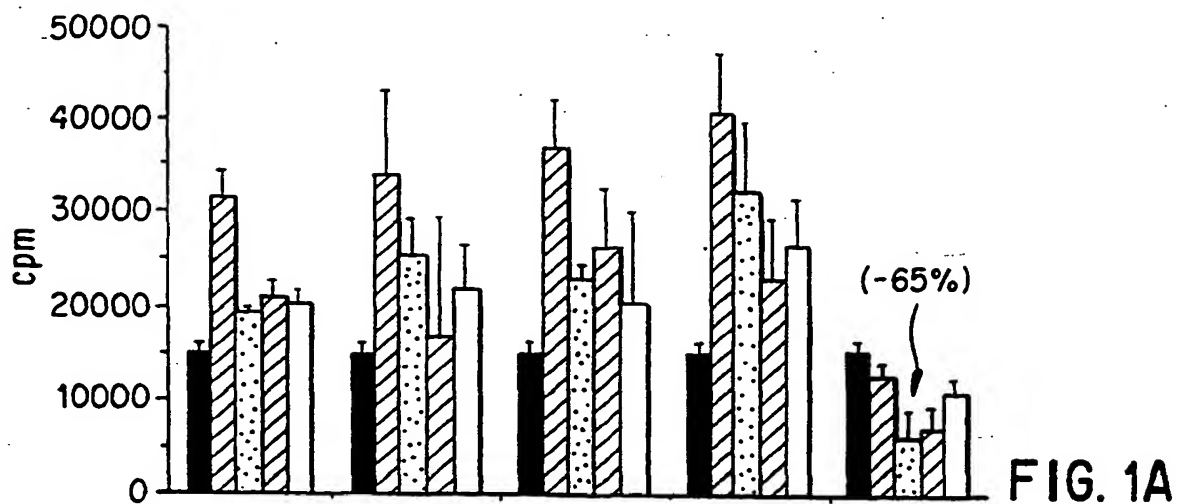
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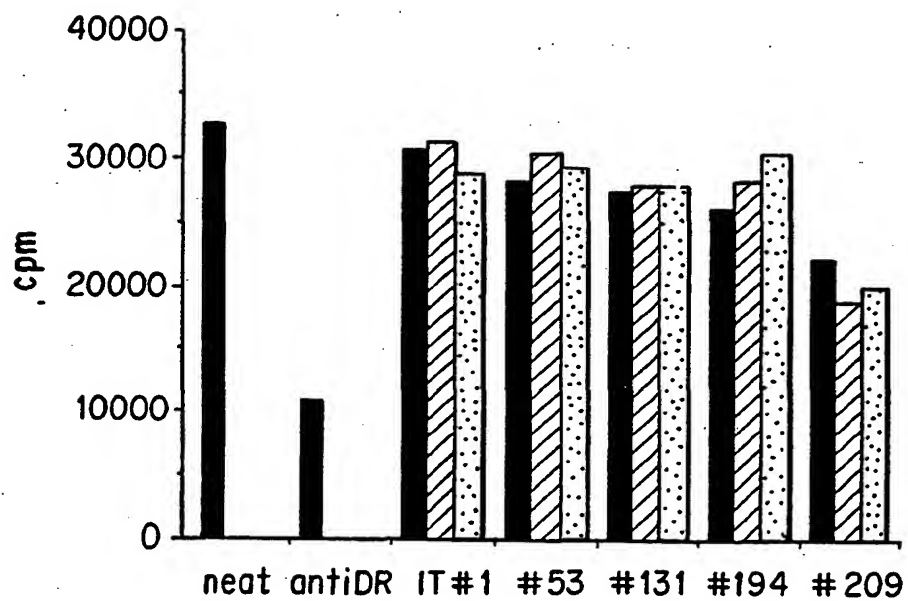


FIG. 2A

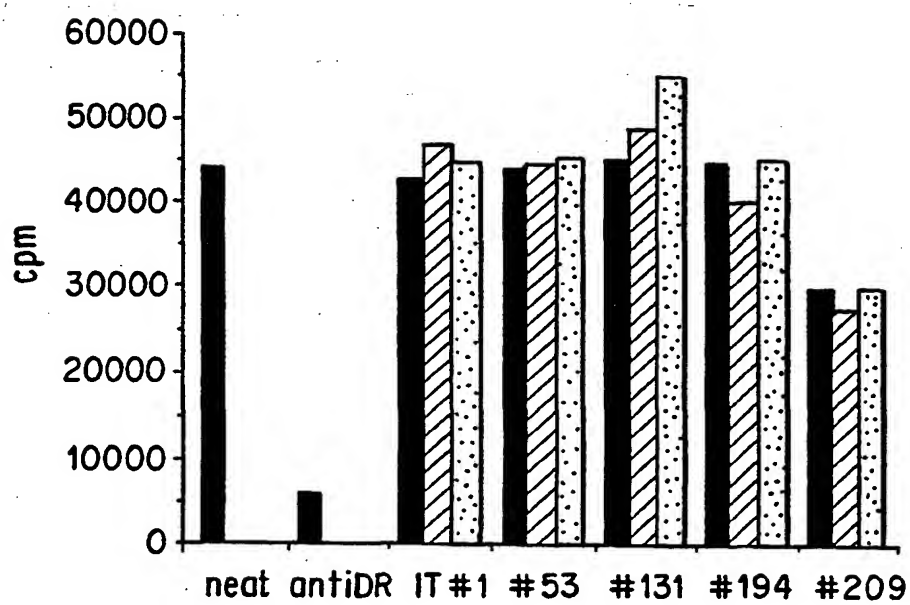


FIG. 2B

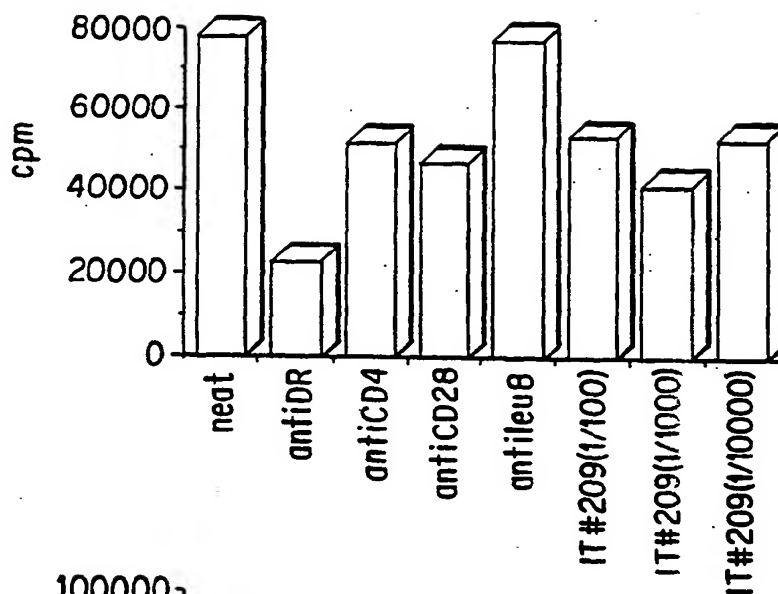


FIG. 3A

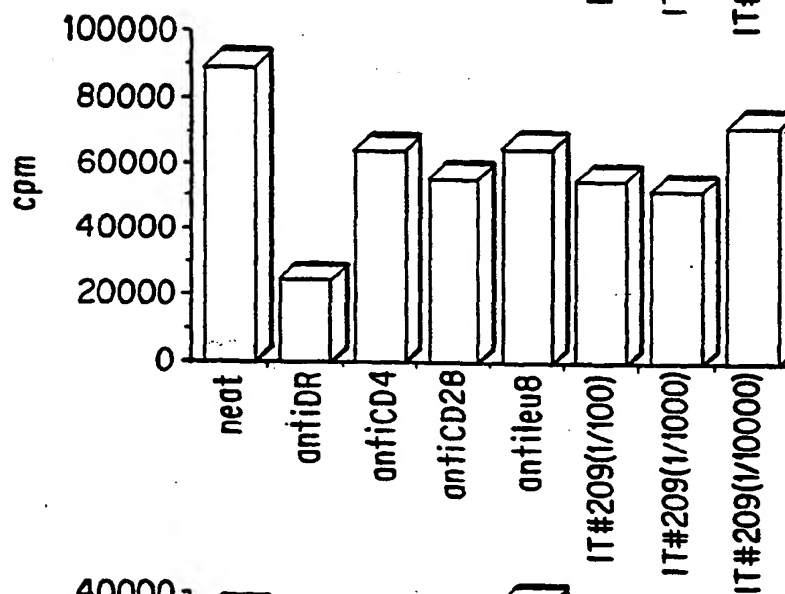


FIG. 3B

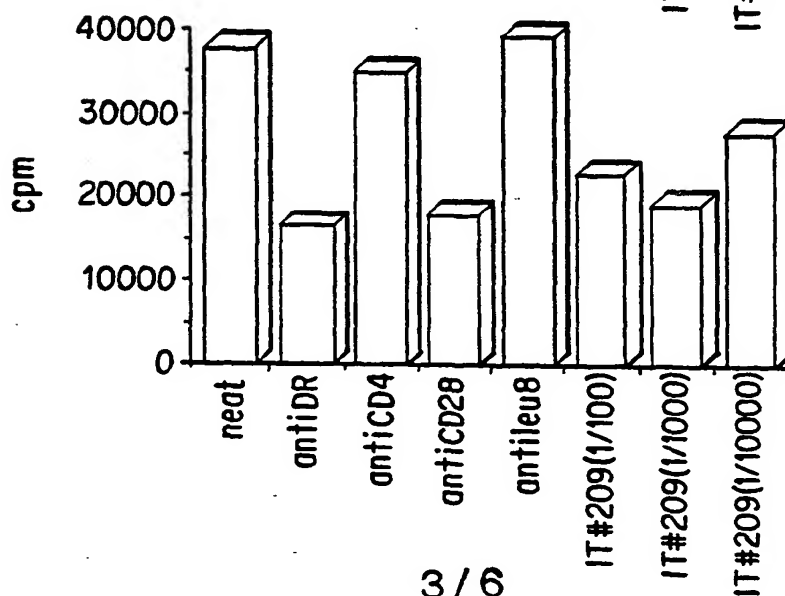


FIG. 3C

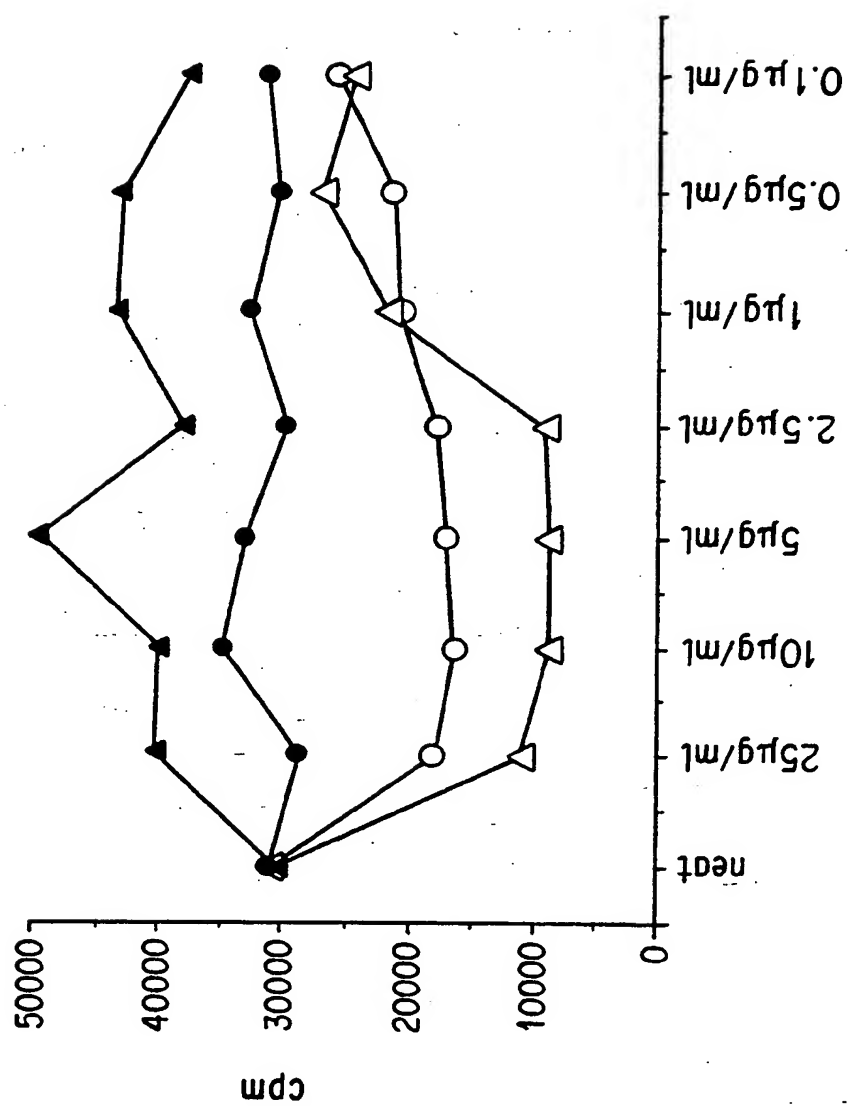


FIG. 4

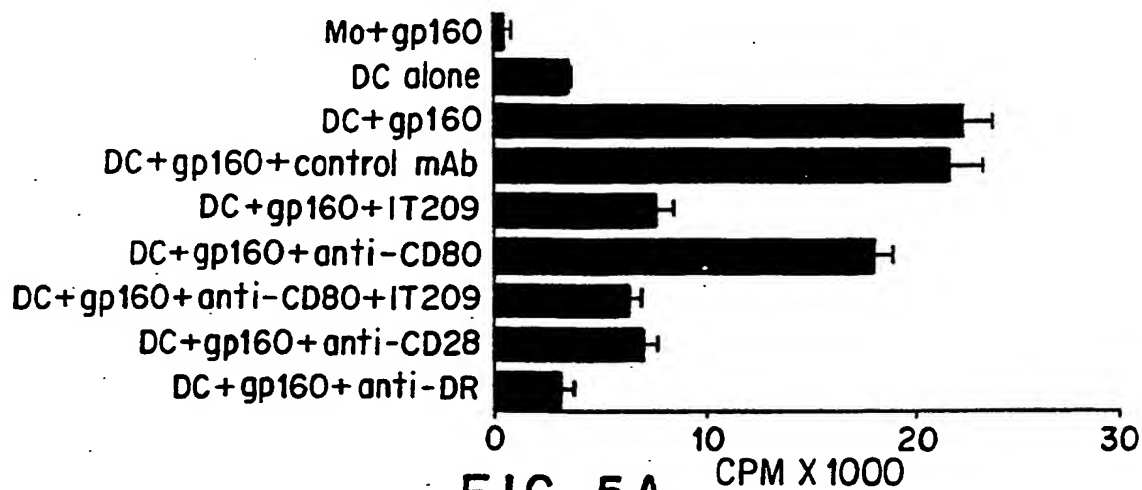


FIG. 5A

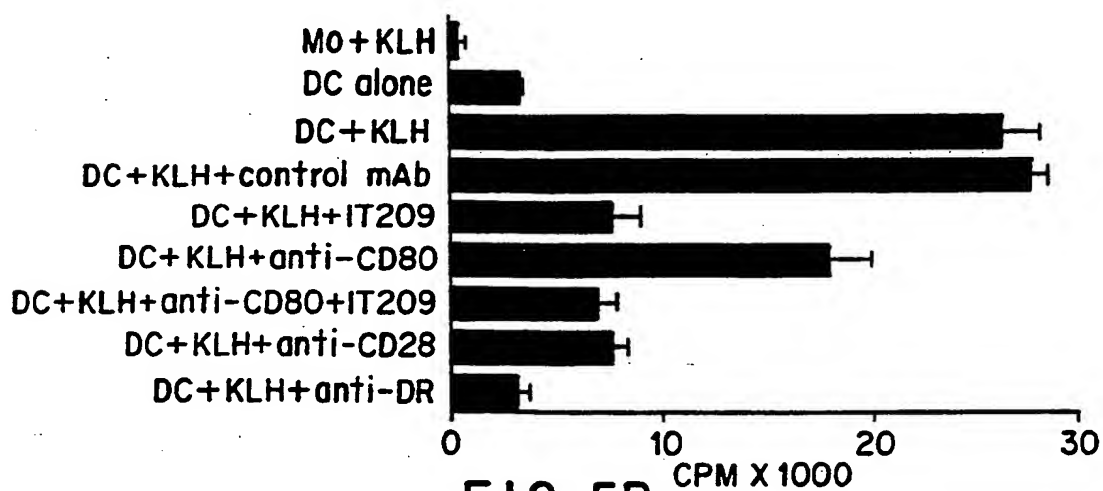


FIG. 5B

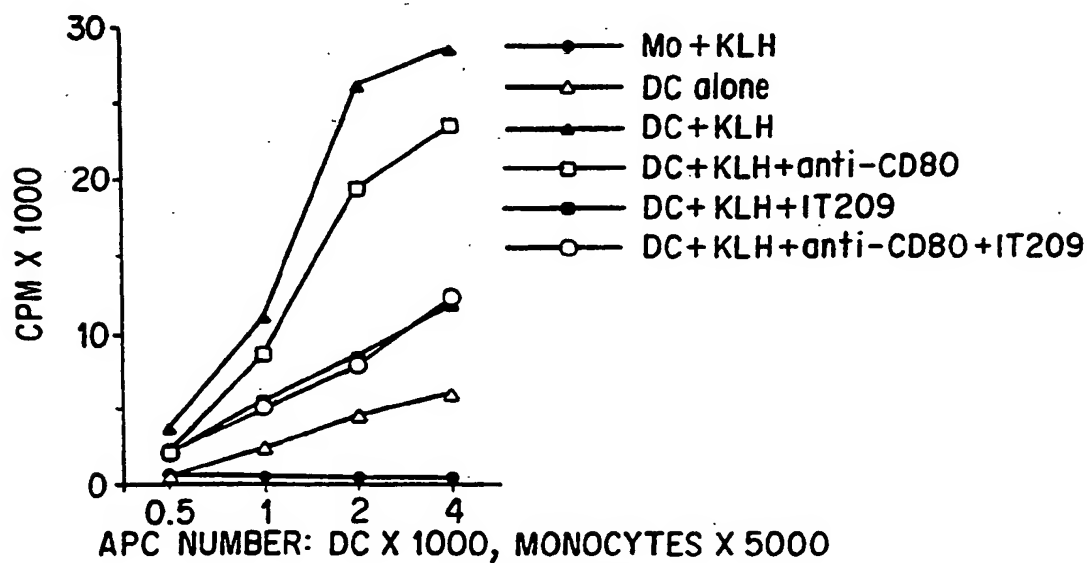


FIG. 5C

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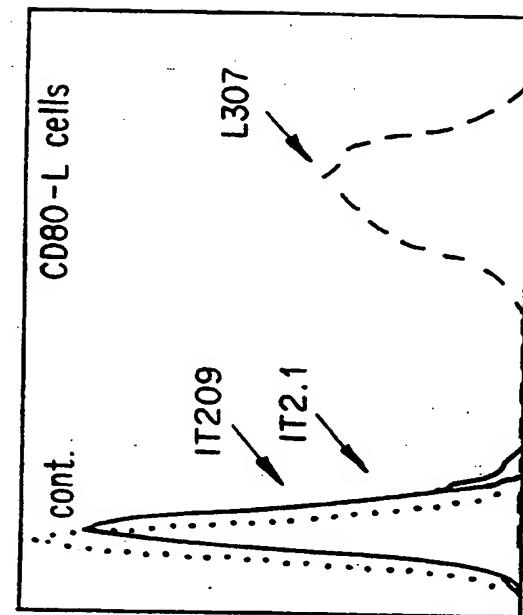


FIG. 6B

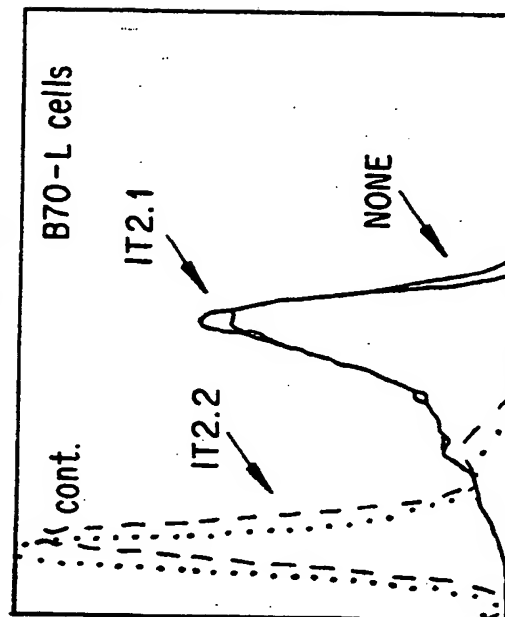


FIG. 6D

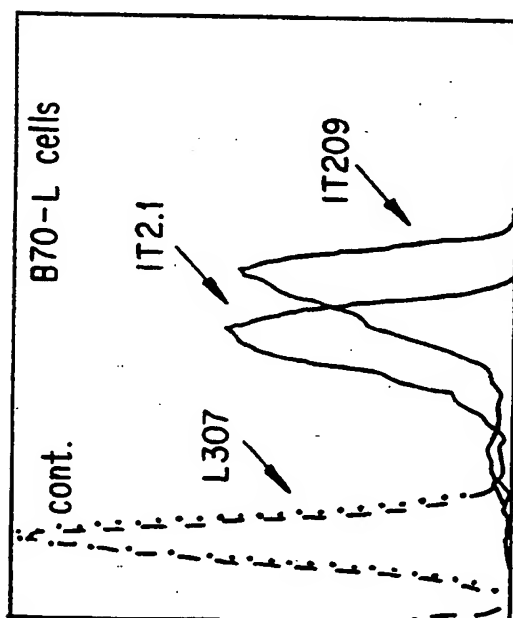


FIG. 6A

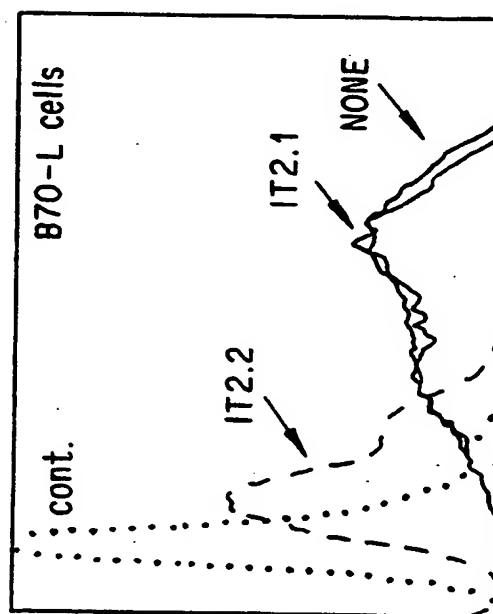


FIG. 6C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13938

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 530/388.2; 435/240, 240.27, 172.2, 70.21, 7.21; 424/152.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.2; 435/240, 240.27, 172.2, 70.21, 7.21; 424/152.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, DIALOG, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, VOLUME 87, ISSUED OCTOBER 1990, FREUDENTHAL ET AL., "THE DISTINCT SURFACE OF HUMAN BLOOD DENDRITIC CELLS, AS OBSERVED AFTER AN IMPROVED ISOLATION METHOD", PAGES 7698-7702, SEE PAGE 7701, TABLE 1, LINE 5 AND PAGES 7698-7699.	1 --- 9
X --- Y	NATURE, VOLUME 366, NUMBER 6450, ISSUED 04 NOVEMBER 1993, AZUMA ET AL., "B70 ANTIGEN IS A SECOND LIGAND FOR CTLA-4 AND CD28", PAGES 76-79, SEE ENTIRE DOCUMENT.	1-3, 5, 6 ----- 4, 7-10
Y	CLINICAL CHEMISTRY, VOLUME 27, NUMBER 11, ISSUED 1981, SEVIER ET AL., "MONOCLONAL ANTIBODIES IN CLINICAL IMMUNOLOGY", PAGES 1797-1806, SEE PAGE 1801.	7, 8

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

03 FEBRUARY 1995

Date of mailing of the international search report

03 MAR 1995

Name and mailing address of the ISA/US
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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US94/13938

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. GODING, "MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE", PUBLISHED 1986 BY ACADEMIC PRESS, PAGES 125-133, SEE PAGES 125-133.	6-8

INTERNATIONAL SEARCH REPORT

International application N .

PCT/US94/13938

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07K 16/28; C12N 5/20, 15/06, 5/08; C12P 21/08; G01N 33/53, 33/535; A61K 39/395